EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016. Scientific opinion on Flavouring Group Evaluation 400 (FGE.400): 3-(1- ((3,5-dimethylisoxazol-4-yl)methyl)-1H-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione

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Scientific opinion on Flavouring Group Evaluation 400 (FGE.400): 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)

Abstract

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) of EFSA was requested to deliver a scientific opinion on the implications for human health of the flavouring substance 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione [FL-no: 16.127], in the Flavouring Group Evaluation 400 (FGE.400), according to Regulation (EC) No 1331/2008 of the European Parliament and of the Council. The substance has not been reported to occur in natural source materials of botanical or animal origin. It is intended to be used as a flavour modifier in specific categories of food. There is no safety concern with respect to genotoxicity. A 90-day dietary administration study in rats showed no adverse effects for doses up to 100 mg/kg body weight (bw) per day, providing an adequate margin of safety. Developmental toxicity was not observed in a study with rats at dose levels up to 1,000 mg/kg bw per day. The Panel concluded that [FL-no: 16.127] is not expected to be of safety concern at the estimated levels of intake. This conclusion applies only to the use of the substance as a flavour modifier and when used at levels up to those specified for various foods in different food categories.

Keywords: (3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione), [FL-no: 16.127], CAS no 1119831-25-2

Requestor: European Commission

Question number: EFSA-Q-2012-00871

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Summary

Following a request from the European Commission (EC), the European Food Safety Authority (EFSA) Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the implications for human health of a chemically defined flavouring substance used in or on foodstuffs in the Member States. In particular, EFSA was requested to evaluate 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione, in the Flavouring Group Evaluation 400 (FGE.400) using the Procedure as referred to in Regulation (EC) No 1334/2008 of the European Parliament and of the Council.

The flavouring substance 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2,4-dione [FL-no: 16.127], contains a number of structural elements common to different chemical groups listed in Annex I to Commission Regulation (EC) No 1565/2000[1] but cannot be adequately covered by any existing FGE. Consequently, the Panel decided to assess this substance on its own.

3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione has not been reported to occur in natural source materials of botanical or animal origin. There are no reports of its detection in processed foods.

Specifications

Specifications including complete purity criteria and identity for the material of commerce have been provided and considered adequate. The candidate substance does not possess chiral centres or geometrical isomers.

The information provided on the manufacturing process, the composition and the stability of the flavouring substance was considered sufficient.

Use and exposure

3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione is intended to be used as a modifier[2] of the bitter taste of specific food categories.

The cumulative dietary exposure to the candidate substance has been estimated using the added portions exposure technique (APET). It is calculated to be 850 μg/capita per day (14 μg/kg body weight (bw) per day for a 60-kg adult) and 536 μg/capita per day (36 μg/kg bw per day for a 15-kg 3-year-old child).

Although the substance is not intended to be used in food categories specifically intended for infants and young children, these could still be exposed through consumption of foods from the general food categories, which may contain the substance. However, at present, there is no generally accepted methodology to estimate exposure in these age groups resulting from consumption of foods from the general categories.

The highest acute intake of the candidate substance results from the consumption of non-alcoholic beverages containing 8 mg/kg of the candidate substance consumed by a 15-kg 3-year-old child. This results an intake of 4.5 mg/capita per day (or 300 μg/kg bw per day for a 15-kg 3-year-old child).

Absorption, distribution, metabolism and elimination

The absorption, distribution, metabolism and elimination (ADME) studies available for [FL-no: 16.127] indicate that the bioavailability of the compound is 2–4% of the orally administered dose. However, the information on the mass balance and metabolic fate of the substance *in vivo* is incomplete. Therefore, the extent of absorption cannot be estimated from the available data. In blood, mainly some conjugates and a minor amount of oxidised metabolites were observed. *In vitro* studies with microsomes (rat and human) indicate very limited phase I metabolism of the candidate substance.

Genotoxicity

No structural alerts have been identified for [FL-no: 16.127]. In tests carried out *in vitro* and *in vivo*, [FL-no: 16.127] showed no potential to cause gene mutations, structural chromosomal

[1] Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation No 2232/96 of the European Parliament and of the Council. Official Journal of the European Union, L 180, p. 8–16.

[2] Regulation No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation No 1601/91, Regulations No 2232/96 and No 110/2008 and Directive 2000/13/EC. Official Journal of the European Union, L 354, p. 34–50.

aberrations or numerical chromosomal aberrations. The Panel concluded that there is no cause for concern with respect to genotoxicity.

Systemic toxicity

A 90-day systemic toxicity study in the rat has been performed. Dietary administration of [FL-no: 16.127] to CD rats for 13 weeks at doses up to 100 mg/kg bw per day was well tolerated, with the only effect being a slight increase in motor activity which was observed only in the high-dose males. Based on the findings in this study, the no-observed-effect-level (NOEL) was considered to be 30 mg/kg bw per day for males and 100 mg/kg bw per day for females, and the no-observed-adverse-effect-level (NOAEL) was 100 mg/kg per day in both sexes.

Developmental toxicity

In a developmental toxicity study, rats were administered oral doses of 125–1,000 mg/kg bw per day of the candidate substance. There were no differences between the treated and control groups. Therefore, there is no concern for developmental toxicity of [FL-no: 16.127] in rats at dose levels up to 1,000 mg/kg bw per day.

Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 0.3 mg/kg bw for a 3-year-old child. Doses of 2,000 mg/kg bw and probably higher are well tolerated in mice without adverse effects. No significant changes in body weight or apparent signs of toxicity were observed in mice administered this dose during the 48-h study period in an *in vivo* micronucleus assay. This results in a margin of exposure of more than 6×10^3 for children and 1.7×10^4 for adults.

Safety assessment for long-term exposure

The safety of long-term exposure has been evaluated according to the Procedure for the evaluation of chemically defined flavouring substances.

For the substance [FL-no: 16.127], there is no concern in relation to genotoxicity and it can be evaluated through the Procedure.

There is no clear structural/metabolic similarity of the candidate substance to flavouring substances in an existing FGE and accordingly the candidate substance [FL-no: 16.127] has been individually evaluated according to the EFSA Guidance document.

Based on its chemical structure, the substance has been assigned to Cramer class III. The results of studies on metabolism and pharmacokinetics do not allow the conclusion that its metabolites are innocuous. Accordingly, the candidate substance is evaluated via the B-side of the Procedure scheme. Based on the comparison of APET with the Cramer class III threshold, a 90-day study and a developmental toxicity study were required and carried out for this substance.

Overall, the Panel concluded that using the NOAEL obtained from a 90-day dietary study in rats, there is no safety concern for [FL-no: 16.127], when used as a flavour modifier at the estimated level of dietary exposure calculated using the APET approach and based on the use levels in food as specified in Appendix C. An adequate margin of safety of over 7,000 for adults and 2,000 for 3-year-old children has been estimated.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The use of flavourings is regulated under Regulation (EC) No 1334/2008[3] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[4]. Regulation (EC) No 1331/2008 shall apply for the evaluation and approval of flavouring substances which are not covered by the evaluation programme provided for in Article 4 of Regulation (EC) No 2232/96[5].

The Commission has received an application for an authorisation of a new flavouring substance 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione.

In order for the Commission to be able to consider its inclusion in the Union list of flavourings and source materials, the European Food Safety Authority (EFSA) should carry out a safety assessment of this substance.

1.1.1. Terms of Reference as provided by the European Commission

The European Commission (EC) requests EFSA to carry out a safety assessment on 3-(1-((3, 5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione [FL-no: 16.127] as a flavouring substance in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

1.2. Interpretation of the Terms of Reference

The present scientific opinion Flavouring Group Evaluation 400 (FGE.400) covers the safety assessment of 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-imidazolidine-2,4-dione [FL-no: 16.127]. This substance will be evaluated as a flavour modifier [cf. Regulation (EC) No: 1334/2008].

2. Assessment

2.1. Identification of the substance

The name of the flavouring substance is 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione, Flavour Information System (FLAVIS)-number: [FL-no: 16.127], Chemical Abstract Service (CAS) no: 1119831-25-2.

2.2. Existing authorisations and evaluations

The substance [FL-no: 16.127] has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) to be of no safety concern (June, 2012 – JECFA No 2161) and has a Flavor and Extract Manufacturers Association (FEMA) GRAS status (FEMA 4725).

2.3. Manufacturing process

2.3.1. Source material

The candidate substance is chemically synthesised. Information on the source materials (starting chemicals, intermediates, reagents and process solvents) has been provided. This information was classified as confidential by the applicant, but is available to EFSA.

[3] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. Official Journal of the European Communities. L 354, 31.12.2008, p. 34–50.

[4] EC (European Commission), 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. Official Journal of the European Communities. OJ L 267, 2.10.2012, p. 1–167.

[5] OJ L 299, 23.11.1996, p. 1.

2.3.2. Genetically modified organism

Not applicable.

2.3.3. Production process

The principles of the synthesis have been provided. Information on the production process was classified as confidential by the applicant, but is available to EFSA.

Considering the starting materials and the employed reaction and purification steps, the Panel concluded that the manufacturing process would not raise safety concern.

2.4. Specifications

The specifications of the candidate flavouring substance are detailed in Table 1.

2.4.1. Chemical name

IUPAC: 3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione.

CAS: 2,4-Imidazolidinedione, 3-[1-[(3,5-dimethyl-4-isoxazolyl)methyl]-1*H*-pyrazol-4-yl]-1-[(3-hydroxy phenyl) methyl].

2.4.2. Identification numbers

CAS no: 1119831-25-2
FLAVIS-no: [FL-no: 16.127]
FEMA GRAS: 4725

2.4.3. Chemical and structural formula, molecular weight

Chemical formula: $C_{19}H_{19}N_5O_4$
Molecular weight: 381.38 Da

2.4.4. Physical form/odour

White to cream-coloured odourless powder.

2.4.5. Solubility data

The following maximum concentrations were obtained at room temperature:

Water (pH 7.1; phosphate buffer): 88 mg/l.
Ethanol: > 9,500 mg/l.
Dimethyl sulfoxide: > 38,000 mg/l.
The Panel calculated a log $P_{o/w}$ of 0.92.

2.4.6. Identity tests

Nuclear magnetic resonance (NMR), ultraviolet (UV), infrared spectroscopy (IR) and high-performance liquid chromatography (HPLC) data have been provided.

2.4.7. Purity/minimum assay value

Minimum assay value: 99.0% by HPLC/UV.
Ethanol and ethyl acetate: ≤ 0.2% by gas chromatography (GC) and NMR.

2.4.8. Impurities

No impurities were detected by HPLC (limit of detection: 0.1%). Analytical data were provided on the levels of ethanol (0.15–0.17% by weight) and ethyl acetate (0.15% by weight) in three commercial batches. No other impurities (byproducts or intermediates, reagents and solvents used in the production process) were observed.

2.4.9. Physical parameters

Melting point: 145–150°C at 986 hPa. According to the industry, the substance [FL-no: 16.127] exhibits crystal polymorphism which can explain the rather wide melting point range (Flavour Industry, 2013).

2.4.10. Configuration

The candidate substance does not possess any asymmetric centres. No geometric isomers are possible. No positional isomers were observed by NMR spectroscopy.

2.4.11. Stability and decomposition products

2.4.11.1. Stability under aqueous conditions

The hydrolytic stability of [FL-no: 16.127] was evaluated in aqueous buffer solutions at various time points over a range of pH values and temperatures by means of liquid chromatography/mass spectrometry (LC/MS). At room temperature, [FL-no: 16.127] was stable for at least 24 h at pH 2.8, 5.0 and 7.1 with recoveries ranging from 104% to 106%. At 60°C, [FL-no: 16.127] was stable for at least 28 days at pH 6.0 (113% recovery), whereas at pH 7.1, ~ 81% remained after 28 days. At 100°C, [FL-no: 16.127] was stable for at least 24 h at pH 2.8 and 5.0, whereas at pH 7.1, 68% of [FL-no: 16.127] remained after 24 h.

Upon heating of [FL-no: 16.127] in phosphate buffer (pH 7.1) at 100°C for 24 h, a number of degradation products were formed (Figure 1).

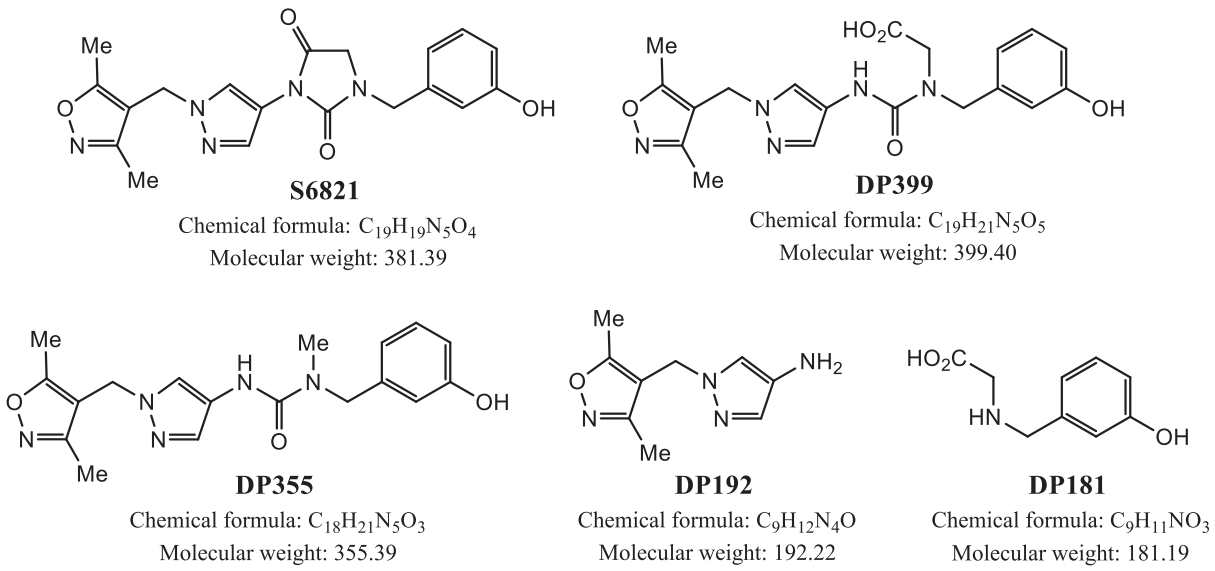


Figure 1: Proposed structures of degradation products upon heating of [FL-no: 16.127] (synonymous to S6821) in phosphate buffer (pH 7.1) at 100°C for 24 h

The stability of [FL-no: 16.127] in aqueous products was also evaluated in typically heat-processed beverages similar to those intended to be marketed. The studies included, for example, the simulation of coffee beverages manufacturing by applying UHT aseptic processing parameters (140°C for 30 s) or involving packaging in gusseted retort pouches and processing in steam/air overpressure to obtain commercial sterility. In all cases, the analytical results demonstrated no significant degradation of [FL-no: 16.127].

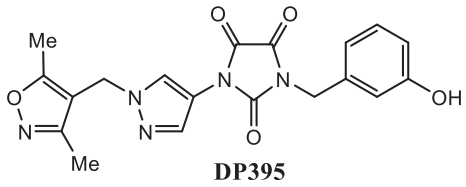
In summary, it was shown that in aqueous solutions the pH is the critical factor for the stability of [FL-no: 16.127]. The flavouring substance exhibits much higher stability in a pH range from 2.8 to 6.0 compared to neutral or basic conditions. According to the applicant, it is recommended that [FL-no: 16.127] should be used in aqueous products at a pH below 6.0 or that the duration of the heating used for processing should be controlled to ensure its stability. The Panel noted that based on the stability tests conducted at 60°C and 100°C, no significant degradation in beverages stored for several months at room temperature is to be expected.

2.4.11.2. Stability under dry conditions

Investigation of the thermal stability of [FL-no: 16.127] as a dry powder via LC/MS demonstrated that the flavouring substance was found to be stable at 100°C for at least 24 h (108% recovery); 76% of the substance [FL-no: 16.127] remained after 4 h at 200°C, whereas 60% of the material remained unchanged after 24 h at this temperature.

As a follow-up to the studies at 200°C, the dry powder stability was evaluated at intermediate temperatures, 150°C and 175°C after heating for 0, 1, 5 and 24 h. In this study, 100% of the compound remained intact after 24 h at 150°C and 95% remained after 24 h at 175°C.

When [FL-no: 16.127] was heated as a dry powder, the following oxidation product was identified (Figure 2):



DP395

Chemical formula: $C_{19}H_{17}N_5O_5$

Molecular weight: 395.37

Figure 2: Structure of the major oxidation product DP395 formed upon heating of [FL-no: 16.127] as dry powder

2.4.11.3. Stability in model foods

When evaluated in key product prototypes, [FL-no: 16.127] was found to be stable in model cakes (95–105% recovery), cookies (103–109% recovery) and hard candies (111–119% recovery) under typical processing conditions (204°C for 8 min, 177°C for 30 min and 149°C for 30 min, respectively). The concentrations of the thermal oxidation product DP395 and of the hydrolytic degradation product DP192 were below the respective limits of detection (LOD) in all products (LODs for DP395 are 0.05 mg/kg in cakes, 0.12 mg/kg in cookies and 0.13 mg/kg in candies; LODs for DP192 are 0.29 mg/kg in cakes and cookies, and 0.02 mg/kg in candies). The hydrolysis product DP399 was formed at low levels in cakes and cookies (0.14 mg/kg in cakes and 0.58 mg/kg in cookies). The substance DP399 could not be detected in candies (LOD = 0.15 mg/kg).

2.4.11.4. Interaction with food components

Trials in which the stability of [FL-no: 16.127] was assessed in different aqueous media as would be encountered in food and beverage applications did not indicate any chemical interaction with other food components.

2.4.12. Particle size

As [FL-no: 16.127] is produced as a solid powder, the particle size and particle size distribution have been measured.[6] The particle size and particle size distribution measured by light scattering are such that the D(50) = 25 μm and D(90) = 69 μm.

To determine whether the particle falls under the definition of nanomaterial, the specific surface area has been measured through the Brunauer–Emmett–Teller (BET) isotherm according to a method described by Brunauer et al. (1938), using an external laboratory.

The findings were:

Specific surface area (single-point determination): 0.24 m^2/g; specific surface area: 0.25 m^2/g

Based on the proposed definition of a nanomaterial by the EC,[7] it is concluded that the candidate substance is not a nanomaterial.

2.4.12.1. Conclusion on specifications and stability

The Panel considered the compositional data and the information on the stability of the flavouring substance as sufficient.

[6] As required by Article 10(c) of Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

[7] Commission Recommendation 2011/696/EU of 18 October 2011 on the definition of nanomaterials. Published in the Official Journal of the European Union L257/38–40 on 20.10.2010 (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011H0696&from=EN).

2.5. Structural/metabolic similarity to substances in an existing FGE (Appendix B)

The candidate substance [FL-no: 16.127] has a number of structural elements common to different chemical groups listed in Annex I to Commission Regulation (EC) No 1565/2000[8]. However, the structure of [FL-no: 16.127] does not allow its integration into one of the existing FGEs. Studies on its metabolism suggest that it is not transformed into substances that fit into existing FGEs; however, the information on the metabolic fate of the substance is incomplete.

As none of the already evaluated substances has been identified with sufficient structural similarity to the candidate substance, the evaluation of this FGE follows the procedure for individual substances, described in the EFSA guidelines (EFSA CEF Panel, 2010) (See Appendix A).

2.6. Exposure assessment (Appendix C)

All data necessary for the calculation of normal and maximum occurrence levels for refined subcategories of foods and beverages are reported in Appendix C.

2.6.1. Natural occurrence

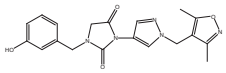
The candidate substance 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2,4-dione is a synthetic substance. It has not been reported to have been identified in natural vegetable or animal source materials. It has not been reported to have been identified in unprocessed or processed food.

2.6.2. Non-food sources of exposure

The candidate substance could conceivably be used to modify the bitterness of pharmaceuticals. No information on this use is presently available. According to the information provided by the applicant, this is not anticipated.

No information has been provided on possible exposure of the candidate substance from use in cosmetics and detergents.

Table 1: Specification summary of the substances in the Flavouring Group Evaluation 400

FL-no JECFA no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b] Others[c]	Boiling point, °C[d] Melting point, °C ID test Assay minimum	Refrac. index[e] Spec. gravity[f]	EFSA comments
16.127 2161	3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2,4-dione		4725 – 1119831-25-2	Odourless, solid $C_{19}H_{19}N_5O_4$ 381.38	Insoluble Slightly soluble Soluble	– 145–150°C IR, UV, NMR, MS 99%	– –	

FL-no: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; EU: European Union; FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; ID: identity; IR: infrared spectroscopy; MS: mass spectrometry; NMR: nuclear magnetic resonance; UV: ultraviolet.
(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95% ethanol, if not otherwise stated.
(c): Solubility in dimethyl sulfoxide.
(d): At 1013.25 hPa (1 atm), if not otherwise stated.
(e): At 20°C, if not otherwise stated.
(f): At 25°C, if not otherwise stated.

[8] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8–16.

2.6.3. Chronic dietary exposure

The exposure assessment to be used in the Procedure for the safety evaluation of the candidate substance is the chronic added portions exposure technique (APET) estimate (EFSA CEF Panel, 2010). The chronic APET has been calculated for adults and children (see Table 2), and these values, expressed per kg body weight (bw), will be used in the Procedure (see Appendices C and G).

Although the substance is not intended to be used in food categories specifically intended for infants and young children, these could still be exposed through consumption of foods from the general food categories, which may contain the substance. However, at present, there is no generally accepted methodology to estimate exposure in these age groups resulting from consumption of foods from the general categories.

Table 2: Chronic dietary exposure estimate

FL-no:	Chronic APET	Added[(a)] (mg/kg bw per day)	Other dietary sources[(b)] (mg/kg bw per day)	Combined[(c)] (mg/kg bw per day)
	Use level	Normal	Average	Normal
16.127	Adults[(d)]	0.014	0	0.014
	Children[(e)]	0.036	0	0.036

APET: added portions exposure technique; bw: body weight.
(a): APET added is calculated on the basis of the amount of flavour added to a specific food category.
(b): APET other dietary sources is calculated based on the natural occurrence of the flavour in a specified food category.
(c): APET combined is calculated based on the combined amount of added flavour and naturally occurring flavour in a specified food category.
(d): For the adult APET calculation, a 60-kg person is considered representative.
(e): For the child APET calculation, a 3-year-old child with a 15-kg bw is considered representative.

2.6.4. Acute dietary exposure

The calculation was based on the maximum use levels and large portion size – i.e. 3 times normal portion size (see Appendix C).

Although the substance is not intended to be used in food categories specifically intended for infants and young children, these could still be exposed through consumption of foods from the general food categories, which may contain the substance. However, at present, there is no generally accepted methodology to estimate exposure in these age groups resulting from consumption of foods from the general categories.

Table 3: Acute dietary exposure estimate

FL-no:	Acute APET	Added[(a)] (mg/kg bw per day)	Other dietary sources[(b)] (mg/kg bw per day)	Combined[(c)] (mg/kg bw per day)
	Use level	Normal	Average	Normal
16.127	Adults[(d)]	0.12	0	0.12
	Children[(e)]	0.30	0	0.30

APET: added portions exposure technique; bw: body weight.
(a): APET added is calculated on the basis of the amount of flavour added to a specific food category.
(b): APET other dietary sources is calculated based on the natural occurrence of the flavour in a specified food category.
(c): APET combined is calculated based on the combined amount of added flavour and naturally occurring flavour in a specified food category.
(d): For the adult APET calculation, a 60-kg person is considered representative.
(e): For the child APET calculation, a 3-year-old child with a 15 kg bw is considered representative.

2.6.5. Cumulative dietary exposure

2.6.5.1. Structurally and metabolically related flavouring substances

There are no other flavouring substances structurally and metabolically related to [FL-no: 16.127] or potentially relevant non-food sources.

2.7. Exposure compared to the threshold of toxicological concern (TTC)

Table 4: Summary table on calculated chronic APET and threshold of concern

	Substance FL-no:	Structural class	Threshold of concern µg/person per day	Threshold of concern x 10 µg/person per day	Add APET µg/person per day	Add APET µg/kg bw per day
Adult	[16.127]	III	90	900	850	14
Child	[16.127]	III	90	900	535	36

APET: added portions exposure technique.

By comparison of the APET exposure estimate with the TTCs and TTCs × 10 (see Table 4), it follows from the Procedure (see Appendix A) that for the evaluation of the candidate flavouring substance the results of a 90-day oral toxicity study and a developmental toxicity study are necessary (see Appendix A). These studies have been submitted by the applicant.

2.8. Absorption, distribution, metabolism and elimination (Appendix D)

The absorption, distribution, metabolism and elimination (ADME) studies available for [FL-no: 16.127] indicate that the bioavailability of the compound is 2–4% of the orally administered dose. However, the information on the mass balance and metabolic fate of the substance *in vivo* is incomplete; therefore, the extent of absorption cannot be estimated from the available data. The studies reported in Appendix D indicate that the primary metabolites of [FL-no: 16.127] in plasma are the glucuronide and sulfate conjugates of [FL-no: 16.127]. Only very small amounts of monohydroxylation products (of the benzyl and isoxazole rings) as well as their corresponding sulfate conjugates were also observed. The study in rat liver microsomes indicated the formation of one monohydroxylated metabolite of [FL-no: 16.127] identified as 3,4-dihydroxybenzyl-[FL-no: 16.127] (identified as S6260 in Figure D.1 in Appendix D). Human liver microsomes formed at least four detectable monohydroxylated metabolites. Two are hydroxylated on the benzyl ring: the 2,5-dihydroxybenzyl derivative of [FL-no: 16.127] and the 3,4-dihydroxybenzyl derivative of [FL-no: 16.127] (identified, respectively, as S6262 and S6260 in Figure D.1 in Appendix D).

2.9. Genotoxicity data (Appendix E)

No structural alerts have been identified for [FL-no: 16.127]. In tests carried out *in vitro* and *in vivo*, [FL-no: 16.127] showed no potential to cause gene mutations, structural chromosomal aberrations or numerical chromosomal aberrations.

The amine hydrolysis product, S6893 (is the same as DP192), did not induce gene mutations in a bacterial reverse mutation assay (AMES test; plate incorporation assay only).

The Panel concluded that there is no cause for concern with respect to genotoxicity.

2.10. Toxicity data (Appendix F)

2.10.1. 90-day dietary systemic toxicity study in rats

A 90-day systemic toxicity study in the rat was performed according to the Organisation for Economic Co-operation and Development ((OECD))-guideline 408 and to good laboratory practice (GLP) (Huntingdon Life Sciences, 2010e). Dietary administration of [FL-no: 16.127] to CD rats for 13 weeks at doses up to 100 mg/kg bw per day was well tolerated, with the only effect being a slight increase in motor activity which was observed only in the high-dose males. As the increase in motor activity was not considered adverse, the Panel considered that the no-observed-adverse-effect-level (NOAEL) was 100 mg/kg bw per day in both sexes.

2.10.2. Developmental toxicity study in rats

In a developmental toxicity study according to the GLP and OECD testing guideline 414, rats were administered oral doses of 125–1,000 mg/kg bw per day of the candidate substance. There were no

differences between the treated and control groups. Therefore, there is no concern for developmental toxicity of [FL-no: 16.127] in rats at dose levels up to 1,000 mg/kg bw per day.

2.11. Safety assessment

2.11.1. Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 0.3 mg/kg bw for a 3-year-old child. Doses of 2,000 mg/kg bw and probably higher are well tolerated in mice without adverse effects. No significant changes in body weight or apparent signs of toxicity were observed in mice administered this dose during the 48-h study period in an *in vivo* micronucleus assay. This gives a margin of exposure of more than 6×10^3 for children and 1.7×10^4 for adults.

2.11.2. Safety assessment for long-term exposure

The safety of long-term exposure will be evaluated according to the Procedure for the evaluation of chemically defined flavouring substances.

Based on the genotoxicity data available, the Panel concluded that for the candidate substance [FL-no: 16.127] there is no safety concern with respect to genotoxicity. Consequently, the substance can be evaluated through the Procedure.

For [FL-no: 16.127], as there is no clear structural/metabolic similarity to other flavouring substances evaluated in an existing FGE, the Panel decided to assess this substance through the Procedure for the evaluation of individual flavouring substances (EFSA CEF Panel, 2010) see Appendix A.

2.11.2.1. Procedure steps

Step 1. Decision on structural class

On the basis of its chemical structure, the substance [FL-no: 16.127] is classified in structure class III (Cramer et al., 1978).

Step 2. Are there data available to demonstrate that metabolites are to be considered innocuous?

The data available do not show that metabolites can be considered to be innocuous. Therefore, [FL-no: 16.127] is evaluated through the B-side of the Procedure.

Step B3. Is the dietary exposure below the respective Cramer class threshold?

The dietary exposure estimate to [FL-no: 16.127] is calculated to be 850 μg/person per day for adults and 536 μg/person per day for children, corresponding to 14 μg/kg bw per day for a 60-kg adult and 36 μg/kg bw per day for a 15-kg child. There is no known exposure from other sources.

The estimated dietary exposure for adults (850 μg/person per day) and for children (536 μg/person per day) exceeds the threshold of concern for structure class III (90 μg/capita per day).

Step B4. Is the dietary exposure below 10 × the respective Cramer class threshold?

The estimated dietary exposure for adults (850 μg/person per day) and for children (536 μg/person per day) is below ten times the threshold for structural class III (i.e. 900 μg/person per day), and according to the Procedure scheme, a 90-day toxicity study and a developmental toxicity study are needed. For the substance [FL-no: 16.127], a NOAEL of 100 mg/kg bw per day (100,000 μg/kg bw per day) from the 90-day oral toxicity study in rats could be identified. A developmental toxicity study has been provided which shows that up to 1,000 mg/kg bw in rats (10-fold higher than the highest exposure in the 90-day toxicity study) there is no indication of developmental toxicity.

The NOAEL of the 90-day oral toxicity study was considered in the risk assessment of the flavouring substance (see Section 2.10.1). The Procedure steps for the candidate substance are shown in schematic form in Appendix A.

2.12. Margin of safety (Table 5)

Table 5: Summary table on calculated margins of safety by toxicity studies

	Study	NOAEL μg/kg bw per day	Add APET μg/kg bw per day	Margin of safety
Adult	90-day dietary study in rats (OECD 408)	100,000	14	> 7,000
Child			36	> 2,000

NOAEL: no-observed-adverse-effect-level; APET: added portions exposure technique; OECD: Organisation for Economic Co-operation and Development.

Based on the Procedure, the Panel concluded that there is no safety concern for the use of [FL-no: 16.127] as a flavour modifier at the estimated level of dietary exposure calculated using the APET approach and based on the use levels in food as specified in Appendix C.

3. Conclusions

The flavouring substance, 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2,4-dione [FL-no: 16.127], contains a number of structural elements common to different chemical groups listed in Annex I to Commission Regulation (EC) No 1565/2000[1] but cannot be adequately covered by any existing FGE. Consequently, the Panel decided to assess this substance on its own.

3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione has not been reported to occur in natural source materials of botanical or animal origin. There are no reports of its detection in processed foods.

Specifications

Specifications including complete purity criteria and identity for the material of commerce have been provided and considered adequate. The candidate substance does not possess chiral centres or geometrical isomers.

The information provided on the manufacturing process, the composition and the stability of the flavouring substance was considered sufficient.

Use and exposure

3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione is intended to be used as a modifier[2] of the bitter taste of specific food categories.

The cumulative dietary exposure to the candidate substance has been estimated using the APET. It is calculated to be 850 μg/capita per day (14 μg/kg bw per day for a 60-kg adult) and 536 μg/capita per day (36 μg/kg bw per day for a 15-kg 3-year-old child).

Although the substance is not intended to be used in food categories specifically intended for infants and young children, these could still be exposed through consumption of foods from the general food categories, which may contain the substance. However, at present, there is no generally accepted methodology to estimate exposure in these age groups resulting from consumption of foods from the general categories.

The highest acute intake of the candidate substance results from the consumption of non-alcoholic beverages containing 8 mg/kg of the candidate substance consumed by a 15-kg 3-year-old child. This results an intake of 4.5 mg/capita per day (or 300 μg/kg bw per day for a 15-kg 3-year-old child).

Absorption, distribution, metabolism and elimination

The ADME studies available for [FL-no: 16.127] indicate that the bioavailability of the compound is 2–4% of the orally administered dose. However, the information on the mass balance and metabolic fate of the substance *in vivo* is incomplete. Therefore, the extent of absorption cannot be estimated from the available data. In blood, mainly some conjugates and a minor amount of oxidised metabolites were observed. *In vitro* studies with microsomes (rat and human) indicate very limited phase I metabolism of the candidate substance.

Genotoxicity

No structural alerts have been identified for [FL-no: 16.127]. In tests carried out *in vitro* and *in vivo*, [FL-no: 16.127] showed no potential to cause gene mutations, structural chromosomal

aberrations or numerical chromosomal aberrations. The Panel concluded that there is no cause for concern with respect to genotoxicity.

Systemic toxicity

A 90-day systemic toxicity study in the rat has been performed. Dietary administration of [FL-no: 16.127] to CD rats for 13 weeks at doses up to 100 mg/kg bw per day was well tolerated, with the only effect being a slight increase in motor activity which was observed only in the high-dose males. Based on the findings in this study, the NOEL was considered to be 30 mg/kg bw per day for males and 100 mg/kg bw per day for females, and the NOAEL was 100 mg/kg bw per day in both sexes.

Developmental toxicity

In a developmental toxicity study, rats were administered oral doses of 125–1,000 mg/kg bw per day of the candidate substance. There were no differences between the treated and control groups. Therefore, there is no concern for developmental toxicity of [FL-no: 16.127] in rats at dose levels up to 1,000 mg/kg bw per day.

Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 0.3 mg/kg bw for a 3-year-old child. Doses of 2,000 mg/kg bw and probably higher are well tolerated in mice without adverse effects. No significant changes in body weight or apparent signs of toxicity were observed in mice administered this dose during the 48-h study period in an *in vivo* micronucleus assay. This results in a margin of exposure of more than 6×10^3 for children and 1.7×10^4 for adults.

Safety assessment for long-term exposure

The safety of long-term exposure has been evaluated according to the Procedure for the evaluation of chemically defined flavouring substances.

For the substance [FL-no: 16.127], there is no concern in relation to genotoxicity and it can be evaluated through the Procedure.

There is no clear structural/metabolic similarity of the candidate substance to flavouring substances in an existing FGE and accordingly the candidate substance [FL-no: 16.127] has been individually evaluated according to the EFSA Guidance document.

Based on its chemical structure, the substance has been assigned to Cramer class III. The results of studies on metabolism and pharmacokinetics do not allow the conclusion that its metabolites are innocuous. Accordingly, the candidate substance is evaluated via the B-side of the Procedure scheme. Based on the comparison of APET with the Cramer class III threshold, a 90-day study and a developmental toxicity study were required and carried out for this substance.

Overall, the Panel concluded that using the NOAEL obtained from a 90-day dietary study in rats, there is no safety concern for [FL-no: 16.127], when used as a flavour modifier at the estimated level of dietary exposure calculated using the APET approach and based on the use levels in food as specified in Appendix C. An adequate margin of safety of over 7,000 for adults and 2,000 for 3-year-old children has been estimated.

Documentation provided to EFSA

1) Flavour Industry, 2013. Unpublished information submitted by Flavour Industry to EFSA and forwarded to FLAVIS Secretariat. A-400 [FL-no: 16.127].
2) Huntingdon Life Sciences, 2009a. S6893: Bacterial Reverse Mutation Screening Test (5 strains).
3) Huntingdon Life Sciences, 2009b. S6821: Toxicity Study by Dietary Administration to CD Rats for 4 Weeks.
4) Huntingdon Life Sciences, 2010a. S6821 Bacterial Reverse Mutation Test.
5) Huntingdon Life Sciences, 2010b. S6821: *In Vitro* Mammalian Chromosome Aberration Test In Human Lymphocytes.
6) Huntingdon Life Sciences, 2010c. S6821: Mouse *In Vivo* Micronucleus Test.
7) Huntingdon Life Sciences, 2010d. S6821 Comparative *In Vitro* Metabolism Using Rat and Human Liver Microsomes.
8) Huntingdon Life Sciences, 2010e. S6821: Toxicity Study by Dietary Administration to CD Rats for 13 Weeks.
9) Huntingdon Life Sciences, 2011. S6821: Pharmacokinetic Study in Rats.

10) Nucro-Technics, 2008a. TA98 And TA100 Reverse Mutation Test Of S6821.
11) Nucro-Technics, 2008b. Acute Oral Toxicity Study with S6821 in Rats.
12) Senomyx, 2010. *In Vivo* Metabolism of S6821 following a Single Oral Dose to Male Sprague-Dawley Rats.
13) Senomyx, 2016. Responses to Request for Additional Information Application for Authorisation of a Flavouring substance from FGE.400 submitted under Commission Implementing Regulations (EC) No 872/2012, (EC) No 234/2011 and (EC) No 1334/2008 of the European Parliament and Council EFSA-Q-2012-00871.
14) WIL Research 2015a. An Oral (Gavage) Dose Range-finding Developmental Toxicity Study of S6821 in Rats. (WIL Study No. 884036).
15) WIL Research 2015b. An Oral (Gavage) Developmental Toxicity Study of S6821 in Rats. (WIL Study No. 884037).

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Abbreviations

ADME	absorption, distribution, metabolism and elimination
APET	added portions exposure technique
AUC	area under the curve
bw	body weight
CAS	Chemical Abstract Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
EC	European Commission
FAO	Food and Agriculture Organization of the United Nations
FDA	US Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GC	gas chromatography
GD	gestation day
GLP	good laboratory practice
GMO	genetically modified organisms
GSFA	General Standard for Food Additives
HPLC	high-performance liquid chromatography
ID	identity
IR	infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LOD	limit of detection
MS	mass spectrometry
MSDI	maximised survey-derived daily intake
NMR	nuclear magnetic resonance
NOAEL	no-observed-adverse-effect-level
NOEL	no-observed-effect-level

OECD	Organisation for Economic Co-operation and Development
SD	Sprague–Dawley
S9-MIX	A metabolic activation system with rat liver microsome fraction plus cofactors
TTC	threshold of toxicological concern
UHT	ultrahigh temperature
UV	ultraviolet
WHO	World Health Organization

Appendix A – Procedure scheme

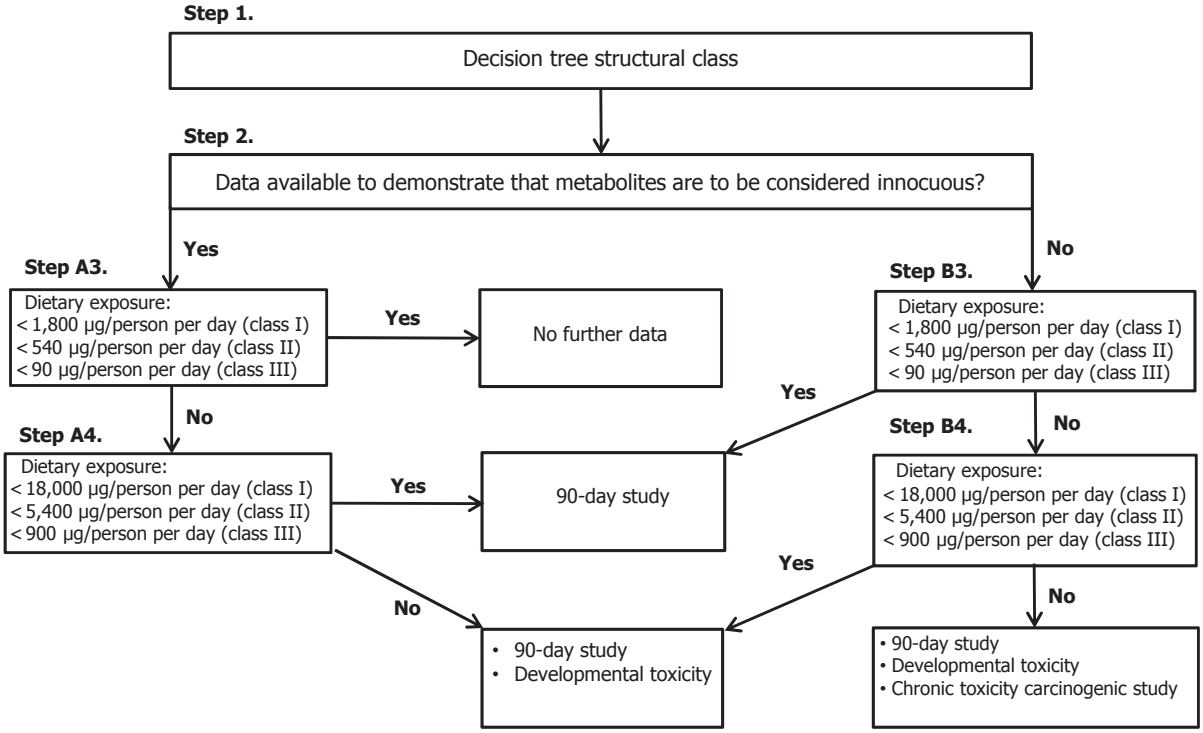


Figure A.1: Individual evaluation of a flavouring substance

Appendix B – Structurally and metabolically related substances

No close structural analogues of the substance [FL-no: 16.127] were identified among the flavouring substances that have already been evaluated by EFSA.

Appendix C – Use levels and exposure calculations

Table C.1: Normal and maximum occurrence levels for subcategories of foods and beverages

	Food categories[a]	Standard portions[b] (g)	Occurrence level as added flavouring (mg/kg)		Occurrence level from other sources[e] (mg/kg)		Combined occurrence level all sources[d] (mg/kg)	
			Normal	Maximum	Normal[c]	Maximum	Normal	Maximum
01.1	Milk and dairy-based drinks	200	1	4			1	4
01.2	Fermented and renneted-milk products (plain), excluding food category 01.1.2 (dairy-based drinks)	200	1	4			1	4
01.3	Condensed milk and analogues (plain)	70	2	8			2	8
01.4	Cream (plain) and the like	15	2	8			2	8
01.5	Milk powder and cream powder and powder analogues (plain)	30	2	8			2	8
01.6	Cheese and analogues	40						
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	125	2	10			2	10
01.8	Whey and whey products, excluding whey cheeses	200	1	4			1	4
02.1	Fats and oils essentially free from water	15						
02.2	Fat emulsions mainly of type water-in-oil	15						
02.3	Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions	15						
02.4	Fat-based desserts, excluding dairy-based dessert products of category 1.7	50						
03.0	Edible ices, including sherbet and sorbet	50	2	4			2	4
04.1.1	Fresh fruit	140						
04.1.2	Processed fruit	125						
04.1.2.5	Jams, jellies, marmalades	30						
04.2.1	Fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed	200						
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter) and nuts and seeds	200						
04.2.2.5	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter)	30						
05.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	40	6	15			6	15
05.1.3	Cocoa-based spreads, including fillings	30						
05.2	Confectionery, including hard and soft candy, nougats, etc., other than 05.1, 05.3 and 05.4	30	8	16			8	16
05.3	Chewing gum	3	10	30			10	30

	Food categories[a]	Standard portions[b] (g)	Occurrence level as added flavouring (mg/kg)		Occurrence level from other sources[e] (mg/kg)		Combined occurrence level all sources[d] (mg/kg)	
			Normal	Maximum	Normal[c]	Maximum	Normal	Maximum
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	35						
06.1	Whole, broken or flaked grain, including rice	200						
06.2	Flours and starches (including soya bean powder)	30						
06.3	Breakfast cereals, including rolled oats	30	8	30			8	30
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soya bean pastas and noodles)	200						
06.5	Cereal and starch-based desserts (e.g. rice pudding, tapioca pudding)	200						
06.6	Batters (e.g. for breading or batters for fish or poultry)	30						
06.7	Precooked or processed-rice products, including rice cakes (Oriental type only)	200						
06.8	Soya bean products (excluding soya bean products of food category 12.9 and fermented soya bean products of food category 12.10)	100						
07.1	Bread and ordinary bakery wares	50						
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	80						
08.1	Fresh meat, poultry and game	200						
08.2	Processed meat, poultry and game products in whole pieces or cuts	100						
08.3	Processed comminute meat, poultry and game products	100						
08.4	Edible casings (e.g. sausage casings)	1						
09.1.1	Fresh fish	200						
09.1.2	Fresh molluscs, crustaceans and echinoderms	200						
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	100						
09.3	Semipreserved fish and fish products, including molluscs, crustaceans and echinoderms	100						
09.4	Fully preserved, including canned or fermented, fish and fish products, including molluscs, crustaceans and echinoderms	100						
10.1	Fresh eggs	100						
10.2	Egg products	100						
10.3	Preserved eggs, including alkaline, salted and canned eggs	100						
10.4	Egg-based desserts (e.g. custard)	125						
11.1	Refined and raw sugar	10						
11.2	Brown sugar excluding products of food category 11.1	10						

	Food categories[a]	Standard portions[b] (g)	Occurrence level as added flavouring (mg/kg)		Occurrence level from other sources[e] (mg/kg)		Combined occurrence level all sources[d] (mg/kg)	
			Normal	Maximum	Normal[c]	Maximum	Normal	Maximum
11.3	Sugar solutions and syrups, and (partially) inverted sugars, including molasses and treacle, excluding products of food category 11.1.3 (soft white sugar, soft brown sugar, glucose syrup, dried glucose syrup, raw cane sugar)	30						
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	30						
11.5	Honey	15						
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	1						
12.1	Salt and salt substitutes	1	25	75			25	75
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1	25	100			25	100
12.3	Vinegars	15	12	25			12	25
12.4	Mustards	15	12	25			12	25
12.5	Soups and broths	200	1	4			1	4
12.6	Sauces and like products	30						
12.7a	Salads 120 g (e.g. macaroni salad, potato salad) excluding cocoa- and nut-based spreads of food categories	120						
12.7b	Sandwich spreads (20 g), excluding cocoa- and nut-based spreads of food categories	20						
12.8	Yeast and like products	1						
12.9	Soybean-based seasonings and condiments	15						
12.9.2	Soybean sauce	15						
12.9.3	Fermented soybean sauce	15						
12.9.1	Fermented soya bean products (e.g. miso)	40	6	20			6	20
12.10	Protein products other than from soybeans	15						
13.2a	Complementary foods for infants and young children: dry instant cereals (with or without milk), including pasta	110						
13.2b	Complementary foods for infants and young children: meat-based or fish-based dinner	170						
13.2c	Complementary foods for infants and young children: dairy-based dessert	110						
13.2d	Complementary foods for infants and young children: vegetables, potatoes, broth, soups and pulses	170						
13.2e	Complementary foods for infants and young children: biscuits and cookies	20						
13.2f	Complementary foods for infants and young children: fruit purée	110						

	Food categories[a]	Standard portions[b] (g)	Occurrence level as added flavouring (mg/kg)		Occurrence level from other sources[e] (mg/kg)		Combined occurrence level all sources[d] (mg/kg)	
			Normal	Maximum	Normal[c]	Maximum	Normal	Maximum
13.2g	Complementary foods for infants and young children: fruit juice	120						
13.2h	Milk for young children	200						
13.3	Dietetic foods intended for special medical purposes (excluding food products of category 13.1 'Infant formulae, follow-up formulae and other formulae for special medical purposes for infants')	200	1	4			1	4
13.4[f]	Dietetic formulae for slimming purposes and weight reduction	200	1	4			1	4
13.5	Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1 (Infant formulae, follow-up formulae and other formulae for special medical purposes for infants), 13.2–13.4 and 13.6	200	1	4			1	4
13.6	Food supplements	5	20	60			20	60
14.1	Non-alcoholic ('soft') beverages (expressed as liquid)	300	2	8			2	8
14.2.1[f]	Beer and malt beverages	300						
14.2.2[f]	Cider and perry	300						
14.2.3[f]	Grape wines	150						
14.2.4[f]	Wines (other than grape)	150						
14.2.5[f]	Mead	150						
14.2.6[f]	Distilled spirituous beverages containing more than 15% alcohol	30						
14.2.7[f]	Aromatised-alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low-alcoholic refreshers)	300						
15.1	Snacks, potato-, cereal-, flour- or starch-based (from roots and tubers, pulses and legumes)	30	8	20			8	20
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	30						
15.3	Snacks – fish based	30	8	20			8	20
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01–15	300						

(a): Most of the categories reported are the subcategories of Codex GSFA (General Standard for Food Additives, available at http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) used by the JECFA in the SPET technique (FAO/WHO, 2008). In the case of category 13.2 (complementary foods for infants and young children), further refined categories have been created so that a specific assessment of dietary exposure can be performed in young children.

(b): In case of foods marketed as powder or as concentrates, occurrence levels must be reported for the reconstituted product, considering the instructions reported on the product label or one of the standard dilution factors established by the JECFA (FAO/WHO 2008):
- 1/25 for powder used to prepare water-based drinks, such as coffee, containing no additional ingredients,
- 1/10 for powder used to prepare water-based drinks containing additional ingredients, such as sugars (ice tea, squashes, etc.),
- 1/7 for powder used to prepare milk, soups and puddings,
- 1/3 for condensed milk.

(c): In order to estimate normal values in each category, only foods and beverages in which the substance is present in significant amount will be considered (e.g. for the category 'fresh fruit' 04.1.1., the normal concentration will be the median concentration observed in all kinds of fruit where the flavouring substance is known to occur).

(d): The normal and maximum combined occurrence levels of the substance will be assessed by the applicant either by adding up occurrence levels from added use to that from other sources or by expert judgment based on the likelihood of their concomitant presence. This will be done both for normal use levels and for maximum use levels.
(e): The data obtained will be an estimate of dietary exposure deriving from all dietary sources, excluding flavourings added to foods and beverages.
(f): The subcategories 14.2.1, 14.2.2, 14.2.3 and 14.2.4 ('alcoholic beverages') and the subcategory 13.4 ('dietetic formulae for slimming purposes and weight reduction') are *a priori* not consumed by children.

Dietary exposure to [FL-no: 16.127] from the consumption of flavoured foods and beverages in adults and children

Chronic dietary exposure

Adults ('added portions exposure technique' (APET)).[9]

On the Basis of Normal Occurrence Level from Added Flavourings

Food subcategories resulting in the highest potential dietary exposure:

Beverage: The maximum intake will be from category 14.1 (non-alcoholic ('soft') beverages). The normal combined occurrence level of 2 mg/kg gives an intake of 600 μg/person per day.

Solid Food: The maximum intake will be from category 1.7 (dairy-based desserts). The normal combined occurrence level of 2 mg/kg gives an intake of 250 μg/person per day.

Total APET: 850 μg/person per day (14.2 μg/kg bw per day for a 60 kg person).

Children (3-year-old child of 15-kg body weight)

The adult portion sizes used in the APET calculations are adjusted by a factor 0.63 to compensate for the lower portion sizes consumed by children.

Food subcategories resulting in the highest potential dietary exposure:

Beverage: The maximum intake will be from category 14.1 (non-alcoholic ('soft') beverages). The normal combined occurrence level of 2 mg/kg gives an intake of 378 μg/child per day (2 mg/kg × 0.63 × 300 g).

Solid Food: The maximum intake will be from category 01.7 (dairy-based desserts). The normal combined occurrence of 2 mg/kg gives an intake of 158 μg/child per day (2 mg/kg × 0.63 × 125 g).

Total APET[10]: 536 μg/child per day (35.7 μg/kg bw per day for a 15-kg child).

Conclusion

The total APET values are 14.2 μg/kg bw per day for 60-kg adults and 35.7 μg/kg bw per day for 15-kg children. In terms of per capita intake, the adult value of 850 μg/day is the higher.

Infants and young children

Although the substance is not intended to be used in food categories specifically intended for infants and young children (food category 13.2a–13.2h), they could still be exposed through consumption of foods from the general food categories, which may contain the substance. However, for the moment, there is no generally accepted methodology to estimate exposure in these age groups resulting from consumption of foods from the general categories.

Acute dietary exposure

Adults

The highest acute intake is assumed to result from the consumption of three portions[11] of non-alcoholic beverages (category 14.1) all containing a maximum concentration of 8 mg/kg of [FL-no: 16.127]. This gives a value of 3 × 300 g × 8 mg/kg = 7.2 mg/capita = 0.12 mg/kg bw for a 60-kg person.

[9] The APET has been calculated based on the occurrence levels in the food subcategories reported in the above Table, with the exclusion of categories 13.2 (complementary foods for infants and young children).
[10] Excluding subcategories 13.4, 14.2.1, 14.2.2, 14.2.3 and 14.2.4. Standard portion sizes for children are obtained by multiplying the adult standard portion sizes by a factor of 0.63.
[11] EFSA Journal 2010; 8(6):1623, Guidance on data submission for flavourings evaluation: In both adults and 3-year-old children, the acute exposure is represented by the consumption of three portions of either a solid food or a beverage, containing the flavouring substance at its maximum occurrence levels.

Children[12]

The highest acute intake is assumed to result from the consumption of three portions (3 × 300 × 0.63 = 567 g) of non-alcoholic beverages (category 14.1) all containing a maximum concentration of 8 mg/kg of [FL-no: 16.127]. This gives an intake value of 567 g × 8 mg/kg = 4.5 mg/capita = 0.3 mg/kg bw for a 15-kg child.

Infants and young children

Although the substance is not intended to be used in food categories specifically intended for infants and young children (food category 13.2a–13.2h), they will also consume food from the general food categories, which may contain the substance. However, for the moment, there is no generally accepted methodology to estimate exposure in these age groups resulting from consumption of foods from the general categories.

Conclusion

The highest acute exposure[13] is assumed to result from the consumption of three portions (3 × 300 × 0.63 = 567 g) of non-alcoholic beverages (category 14.1) all containing a maximum concentration of 8 mg/kg of [FL-no: 16.127]. This gives an intake value of 567 g × 8 mg/kg = 4.5 mg/capita = 0.3 mg/kg bw for a 15 kg 3-year-old child.

Cumulative Dietary Exposure to [FL-no: 16.127]

Any other flavouring substances structurally and metabolically related to [FL-no: 16.127]: none found.

Occurrence levels for structurally and metabolically related substances which have already been evaluated in an FGE: none found.

Potential cumulative dietary exposure within 1 day to flavouring substances structurally and metabolically related to [FL-no: 16.127]: none found.

Potential non-food sources: none found.

Cumulative dietary exposure to [FL-no: 16.127] will therefore be 14.2 μg/kg bw per day for 60-kg adults and 35.7 μg/kg bw per day for 15-kg infants. In terms of per capita intake, the adult value of 850 μg per day is the higher.

[12] Based on the same considerations as for adults but using the special factors used for chronic exposure to infants.
[13] The highest value obtained among adults and children of all ages.

Appendix D – Absorption, distribution, metabolism and elimination

In vivo pharmacokinetics following a single-oral dose to rats

The objective of the study was to investigate the *in vivo* pharmacokinetics of [FL-no: 16.127] following a single-oral administration to rat (Senomyx, 2010).

Male Sprague–Dawley (SD) rats were orally dosed with 100 mg/kg bw of [FL-no: 16.127] and blood samples were collected at three time points (0.5, 2 and 4 h) after dosing (Senomyx, 2010). The plasma samples were analysed by LC/MS/MS with an internal standard 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-5,5-dimethylimidazolidine-2,4-dione.

The maximum concentration of [FL-no: 16.127] in rat plasma was observed at the 0.5-h time point in all the test animals (mean concentration: 105.4 ng/mL, n = 4). The mean concentrations of the glucuronide of [FL-no: 16.127] (S7558) and the sulfate of [FL-no: 16.127] (S5907) in the plasma samples during the 4-h sampling period were in the range of 32–166 ng/mL and 334–555 ng/mL, respectively. The mean concentration of a monohydroxylated metabolite, 2,3-dihydroxybenzyl derivative of [FL-no: 16.127] (S6887), was in the range of 12.6–18.9 ng/mL throughout the observation period. Ions consistent with the presence of metabolites in which the isoxazole ring of [FL-no: 16.127] has undergone oxidation were also seen in the extracted ion chromatograms. Sulfate derivatives of the monohydroxylated metabolites were also observed. The samples were also analysed for the presence of two alternative hydroxylated metabolites (S6260, S6262) and two potential hydrolytic breakdown products (the hydantoin hydrolysis product of [FL-no: 16.127], referred to as S6893; and the hydantoin ring-opened product of [FL-no: 16.127], referred to as S4687), but these were either absent or below the limit of detection (< 1.0 ng/mL).

Conclusion

The primary metabolites of [FL-no: 16.127] in plasma are the glucuronide and sulfate conjugates of unoxidised [FL-no: 16.127]. Minor amounts of monohydroxylation products [FL-no: 16.127] (on the benzyl and isoxazole rings) as well as their corresponding sulfate conjugates were also observed. Metabolite structures are shown in Figures D.1 and D.2.

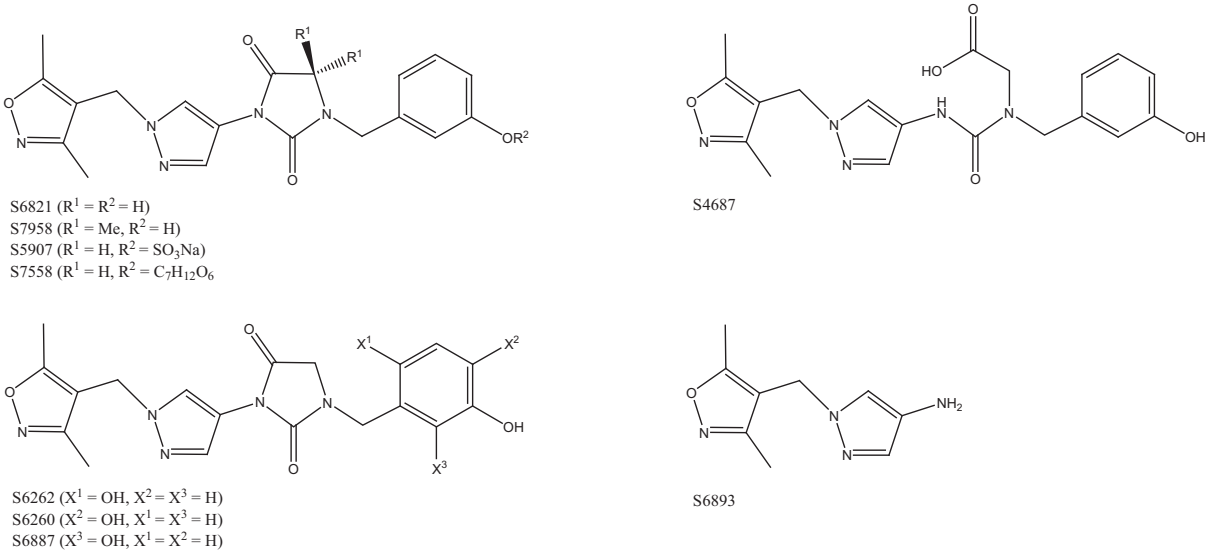


Figure D.1: Metabolically related substances, as indicated by the applicant

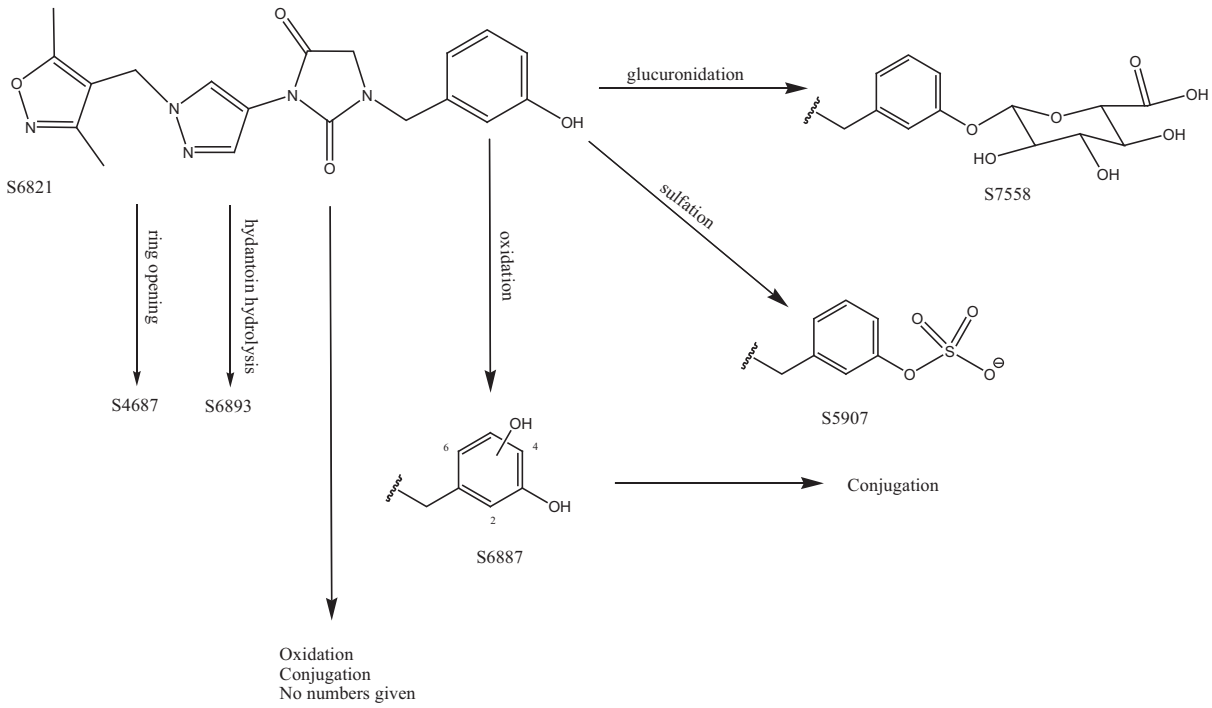


Figure D.2: Proposed metabolic pathways of [FL-no: 16.127]

Pharmacokinetic and metabolite detection study in rats following a single bolus intravenous or oral dose

The objective of the study was to perform pharmacokinetic evaluation of both unchanged test compound concentrations and concentrations of two of its conjugate metabolites (the sulfate S5907 and the glucuronide S7558), in plasma, following single-oral gavage or intravenous administration to rats. (Huntingdon Life Sciences, 2011).

Four groups of six male SD rats were dosed; the first by intravenous bolus administration at a nominal dose of 1 mg/kg bw and the remaining three groups by oral gavage administration at nominal dosages of 10, 30 and 100 mg/kg bw. Blood samples were collected post-dose, over nine time points for the orally dosed groups and ten time points for the intravenously dosed group and centrifuged to obtain plasma for pharmacokinetic analysis. Each group of animals was split into two subgroups of three animals (cohorts) for the purposes of blood sampling. Sampling was alternated between cohorts at each time point, such that only three animals were sampled at each time point. Samples were taken at 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h post-dose with an additional time point at 5 min (0.083 h) for the intravenously treated animals. Plasma samples were then analysed for the concentration of [FL-no: 16.127] and two of its conjugate metabolites (the sulfate S5907 and the glucuronide S7558) using a suitable LC/MS/MS method.

Following intravenous administration of [FL-no: 16.127] to male SD rats, elimination of both the parent substance [FL-no: 16.127] and its glucuronide S7758 was shown to be rapid with terminal half-lives of 0.1 and 0.2 h, respectively. The terminal half-life of the sulfate S5907, however, was longer than that of [FL-no: 16.127] indicating that the rate of formation of this metabolite was not the rate-limiting step in its elimination. Systemic exposure, as reflected by the parameter 'area under the curve' (AUC_{all}), was shown to be highest for metabolite S5907, whereas exposure to [FL-no: 16.127] and metabolite S7558 were similar. The total plasma clearance of [FL-no: 16.127] was slightly lower than the reported cardiac plasma output in rats and the volume of distribution at steady state was less than the reported total body water in rats.

Following oral administration of [FL-no: 16.127] to male SD rats, [FL-no: 16.127] absorption was rapid with a terminal half-life of [FL-no: 16.127] (at the highest dose level) of 4.3 h. The systemic exposure of rats to [FL-no: 16.127] appeared to be characterised by non-linear kinetics. The C_{max} values increased by less than the proportionate dose interval, however, the AUC_{all} values were up to 1.5-fold higher than the values predicted by a linear relationship. The systemic bioavailability of [FL-no: 16.127] following oral administration was low, with the rate and extent to which [FL-no: 16.127]

reaches the systemic circulation (F) being only 2%, 4% and 2% following nominal oral doses of 10, 30 and 100 mg/kg bw, respectively. The terminal half-lives of metabolites S5907 and S7558 ranged between 2.5–4.7 and 1.4–4.2 h, respectively, following oral doses of [FL-no: 16.127] between 10 and 100 mg/kg bw, with the longest half-lives being exhibited at the highest dosage. The C_{max} and AUC values for both metabolites increased with increasing dose over the range 10–100 mg/kg bw [FL-no: 16.127], however, the increases were less than the proportionate dose increment.

Conclusion

The systemic bioavailability of [FL-no: 16.127] following oral administration was low, with percentage values for F of 2%, 4% and 2% following nominal oral doses of 10, 30 and 100 mg/kg bw, respectively, suggesting that systemic exposure may be absorption limited. Following intravenous administration of [FL-no: 16.127] to male SD rats, elimination of both the parent and its glucuronide conjugate (S7758) were shown to be rapid with terminal half-lives of 0.1 and 0.2 h, respectively. The terminal half-life of the sulfate conjugate S5907, however, was longer than that of [FL-no: 16.127], although still rapid being 2.9, 2.5 and 4.7 h, respectively, at these doses. Following oral administration of [FL-no: 16.127] to male rats, [FL-no: 16.127] absorption was rapid with a terminal half-life of 4.3 h (at the highest dose level). The terminal half-lives of metabolites S5907 and S7558 ranged between 2.5–4.7 and 1.4–4.2 h, respectively. Systemic exposure to the parent and metabolites, was shown to increase with increasing dosage, however, the increases in C_{max} were lower than predicted from a linear relationship. Therefore, exposure to [FL-no: 16.127] or its major metabolites was minimal. However, the limited absorption and limited metabolism have not been confirmed by, e.g. experiments in a metabolism cage in which after oral administration, it is shown that over 95% of the dose is excreted unchanged in faeces.

In vitro biotransformation in rat and human liver microsomes

The objective of this study was to investigate and compare the Phase I metabolism of [FL-no: 16.127] using liver microsomes from rat and human (Huntingdon Life Sciences, 2010d).

The Phase I metabolism of [FL-no: 16.127] was investigated using liver microsomes from rat and human. [FL-no: 16.127] (1, 10 and 50 μM) was incubated with pooled-rat and pooled-human liver microsomes (0.5 mg/mL) for up to 120 min. The reaction was stopped by addition of acetonitrile. The samples were centrifuged and the supernatants taken for LC/MS analysis. LC/MS analysis of rat and human liver microsomes incubation samples was carried out on a Waters Symmetry C18 column (150 x 3.9 mm) with 0.1% formic acid and acetonitrile gradient system. The TSQ7000 mass spectrometer was operated in positive ionisation mode.

LC/MS results indicate that rat liver microsomes formed primarily one monohydroxylated metabolite of [FL-no: 16.127] identified as 3,4-dihydroxybenzyl-[FL-no: 16.127] (S6260). Human liver microsomes formed at least four identifiable monohydroxylated metabolites. Two are hydroxylated on the benzyl ring, 2,5-dihydroxybenzyl-[FL-no: 16.127] (S6262) and 3,4-dihydroxybenzyl-[FL-no: 16.127] (S6260). The structures were confirmed by LC/MS comparison to authentic compounds. The other two were monohydroxylated at undetermined positions on the dimethylisoxazole ring as confirmed by product ion mass spectra.

Overall conclusions on absorption, distribution, metabolism and elimination

The ADME studies available for [FL-no: 16.127] indicate that the bioavailability of the compound is 2–4% of the orally administered dose. However, the information on the mass balance and metabolic fate of the substance *in vivo* is incomplete; therefore the extent of absorption cannot be estimated from the available data. The studies reported in this Appendix indicate that the primary metabolites of [FL-no: 16.127] in plasma are the glucuronide and sulfate conjugates of [FL-no: 16.127]. Only very small amounts of monohydroxylation products (of the benzyl and isoxazole rings) as well as their corresponding sulfate conjugates were also observed. The study in rat liver microsomes indicated the formation of one monohydroxylated metabolite of [FL-no: 16.127] identified as 3,4-dihydroxybenzyl-[FL-no: 16.127] (identified as S6260 in Figure D.1 in Appendix D). Human liver microsomes formed at least four detectable monohydroxylated metabolites. Two are hydroxylated on the benzyl ring: the 2,5-dihydroxybenzyl derivative of [FL-no: 16.127] and the 3,4-dihydroxybenzyl derivative of [FL-no: 16.127] (identified, respectively, as S6262 and S6260 in Figure D.1 in Appendix D).

Appendix E – Genotoxicity

Genotoxicity data *in vitro*

Bacterial Reverse Mutation Assay

The candidate substance was tested in *Salmonella* Typhimurium strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2 *uvrA* in accordance with the OECD Guideline 471 (Huntingdon Life Sciences, 2010a). Two independent mutation tests were performed in the presence and absence of liver preparations (S9-mix) from rats treated with phenobarbital and 5,6-benzoflavone. The first test was a standard plate incorporation assay; the second included a preincubation stage.

Concentrations of [FL-no: 16.127] up to 5,000 μg/plate were tested. Other concentrations used were a series of ca. half-$\log_{10}$ dilutions of the highest concentration.

No signs of toxicity were observed towards the tester strains in either mutation test following exposure to [FL-no: 16.127]. No evidence of mutagenic activity was seen at any concentration of [FL-no: 16.127] in either mutation test. The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It was concluded that the candidate substance was not mutagenic to *S.* Typhimurium strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2 *uvrA*, when tested in accordance with regulatory guidelines.

A bacterial reverse mutation test was also conducted on the amine hydrolysis product (S6893 – see Figure 1; Huntingdon Life Sciences, 2009a). The amine hydrolysis product, S6893 (is the same as DP192), tested in *S.* Typhimurium strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2 *uvrA* in the absence and presence of metabolic activation (plate incorporation assay only), did not induce gene mutations.

It was concluded that S6893 was not mutagenic in this bacterial system under the test conditions employed.

Chromosome Aberration Test

An *in vitro* chromosome aberration test was performed on [FL-no: 16.127] in order to investigate its potential to induce structural chromosome aberrations in cultured-human lymphocytes. The experimental design followed the OECD Guideline 473 (Huntingdon Life Sciences, 2010b).

First test: In the absence and presence of S9-mix – 3 h treatment, 18 h recovery. Concentrations tested: 296.6, 494.3 and 823.8 μg/mL. In the absence of S9-mix, appropriate toxicity was achieved at the two highest concentrations (relative mitotic index: 66%). However, in the presence of S9-mix, no significant reduction in the mitotic index was achieved, even at the highest concentration.

Second test: In the absence of S9-mix – 21 h continuous treatment. Concentrations tested: 200, 400 and 600 μg/mL. In the presence of S9-mix (5% v/v) – 3 h treatment, 18 h recovery. Concentrations tested: 1,100, 1,200 and 1,300 μg/mL. Both in the absence and in the presence of S9-mix appropriate toxicity was achieved (relative mitotic index: 70% and 51% at 400 and 600 μg/mL in the absence of S9-mix, respectively; 67% and 51% at 1,200 and 1,300 μg/mL in the presence of S9-mix, respectively).

In the absence of S9-mix, [FL-no: 16.127] caused no statistically significant increases in the proportion of metaphase figures containing chromosomal aberrations, at any concentration, when compared with the solvent control, in either test. In the first test in the presence of S9-mix, no statistically significant increases in the proportion of metaphase figures containing chromosomal aberrations were observed at any concentration. However, no significant reduction in the mitotic index was achieved at the used concentrations; therefore, the results of this test have to be considered as inconclusive. In the second test in the presence of S9-mix, appropriate reduction in the mitotic index was achieved at concentrations higher than those used in the first test. Under the experimental conditions used in the second test in the presence of S9-mix, no statistically significant increases of chromosomal aberrations were observed at any concentration.

No statistically significant increases in the proportion of polyploid cells were observed during metaphase analysis, in either test.

All positive control compounds caused statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9-mix.

It is concluded that [FL-no: 16.127] shows no evidence of causing an increase in the frequency of structural chromosome aberrations in this *in vitro* cytogenetic test system, under the experimental conditions described.

Genotoxicity data *in vivo*

Mouse Micronucleus Test

A mouse micronucleus test was performed with [FL-no: 16.127] in accordance with the OECD Guideline 474 (Huntingdon Life Sciences, 2010c).

The preliminary toxicity test demonstrated that a dose of 2,000 mg/kg bw per day, administered on two consecutive occasions approximately 24 h apart, was tolerated. On the basis of these results, dose levels of 500, 1,000 and 2,000 mg/kg bw per day were selected for the micronucleus test. No substantial differences in toxicity were observed between the sexes; therefore, the main test was performed using male animals only. The vehicle was 1% methylcellulose in purified water. All animals in the vehicle control and test substance dose groups were dosed orally by gavage using a dose volume of 10 mL/kg. The positive control group animals were dosed orally by gavage using a dose volume of 20 mL/kg. The negative control group received the vehicle 1% methylcellulose in purified water and the positive control group received Mitomycin C at 12 mg/kg bw. Bone marrow smears were obtained from animals in the vehicle control and in each of the test substance groups 24 h after administration of the second dose. In addition, bone marrow smears were also obtained from animals in the positive control group 24 h after a single dose. One smear from each animal was examined for the presence of micronuclei in 2,000 polychromatic erythrocytes. The proportion of polychromatic erythrocytes was assessed by examination of at least 1,000 erythrocytes from each animal. A record of the incidence of micronucleated normochromatic erythrocytes was also kept.

No statistically significant increases in the frequency of micronucleated polychromatic erythrocytes and no statistically significant decreases in the proportion of polychromatic erythrocytes were observed in CD1 mice treated with [FL-no: 16.127] at any treatment level, compared to vehicle control values.

The positive control compound, Mitomycin C, produced a statistically significant increase in the frequency of micronucleated polychromatic erythrocytes ($p < 0.01$).

The mean concentrations of [FL-no: 16.127] in test formulations analysed during the study were within the applied limits (100 $\pm$ 10%), confirming the accuracy of the formulation(s).

It is concluded that [FL-no: 16.127] did not show any evidence of causing an increase in the induction of micronucleated polychromatic erythrocytes or bone marrow cell toxicity in male CD1 mice when administered orally by gavage in this *in vivo* test procedure. The test material was not clastogenic and it did not interact with the mitotic spindle.

The Panel noted that bone marrow exposure was not demonstrated in the *in vivo* micronucleus assay. However, as the *in vitro* chromosomal aberration assay was negative, there is no need for further *in vivo* follow-up.

Appendix F – Toxicity

Acute oral toxicity study in rats

This preliminary test was carried out on [FL-no: 16.127] in order to ensure that there are no likely acute toxic effects that would preclude the initial sensory examination of this material at low levels as a flavouring agent (Nucro-Technics, 2008b). Three groups of five female SD rats were tested. Group 1, Group 2 and Group 3 animals were administered dose levels of 5, 15 and 50 mg/kg bw, respectively, at dose concentrations of 0.5, 1.5 and 5.0 mg/mL. The test article was suspended in 1% methyl cellulose and was administered by oral gavage at a dose volume of 10 mL/kg. The animals were observed for an 8-day period after dosing. Body weights were recorded prior to test article administration (day 0), on day 4, day 7 and prior to necropsy on day 8.

No mortality or evidence of toxicity were observed post-dosing or during the 8-day observation period in any of the animals. Animals in each group gained body weight by the end of study. Based on clinical observations and gross necropsy, the test article, [FL-no: 16.127], did not show any evidence of toxicity when administered orally to rats at dose levels of up to 50 mg/kg bw under the aforementioned testing conditions.

28-day rat range-finding dietary toxicity study in rats

A range-finding oral toxicity screening study was carried out in rats on [FL-no: 16.127] in order to provide information for dose selection of the subsequent 90-day toxicity study in rats (Huntingdon Life Sciences, 2009b).

The potential systemic toxicity of [FL-no: 16.127] was evaluated in male and female Crl:CD®(SD) rats (5/sex per group). The study was conducted in compliance with the US FDA Toxicological Principles for the Safety of Food Ingredients (as revised in 2004). The compound was administered in the diet at doses of 0 (control), 10, 30 or 100 mg/kg bw per day for a period of 28 days. Survival, clinical observation, body weight, food consumption, haematology, clinical chemistry, organ weights and macroscopic evaluations of all animals were used to assess potential toxicity. The livers and gross lesions of all animals were subjected to histopathological examination.

The compound induced no treatment-related changes in mortality, clinical observations, body weights, food consumption, haematology or clinical chemistry parameters. Macroscopic examination at necropsy, organ weights and microscopic examination of the liver were unremarkable.

Therefore, dietary administration of [FL-no: 16.127] to CD rats for 4 weeks at doses up to 100 mg/kg bw per day was well tolerated, as there was no evidence of toxicity.

90-day dietary toxicity study in rats

[FL-no: 16.127] (99% pure) was administered in the diet of rats over 90 days in order to evaluate its subchronic toxicity (Huntingdon Life Sciences, 2010e). This study was designed to be in accordance with the OECD Guideline 408 and the guidelines of the US FDA Red Book and was conducted in accordance with the requirements of current, internationally recognised GLP standards.

The potential systemic toxicity of [FL-no: 16.127] was evaluated in male and female Crl:CD(SD) rats (20/sex/group). The compound was administered in the diet at doses of 0 (control), 10, 30, or 100 mg/kg bw per day for a period of 13 weeks. Survival, clinical observation, body weight, food consumption, ophthalmic and physical examinations, haematology, clinical chemistry, urinalysis, motor activity, sensory reactivity and grip strength, organ weights, and macroscopic and microscopic evaluations were determined.

There were no treatment-related changes in mortality, clinical observations, sensory reactivity and grip strength, body weights, food consumption, ophthalmic exams, haematology, clinical chemistry or urinalysis parameters. Macroscopic examination at necropsy, organ weights and microscopic examinations were unremarkable. Motor activity scores (both high and low beam) were slightly increased, when compared to the controls, for males receiving 100 mg/kg bw per day. Males and females receiving 10 and 30 mg/kg bw per day and females receiving 100 mg/kg bw per day were not affected. As there were no other changes that were indicative of toxicity in these animals, the increase in motor activity was not considered adverse.

In conclusion, dietary administration of [FL-no: 16.127] to CD rats for 13 weeks at doses up to 100 mg/kg bw per day was well tolerated, with the only effect being a slight increase in motor activity which was observed only in the high-dose males. Based on the findings in this study, the NOEL was considered to be 30 mg/kg bw per day for males and 100 mg/kg bw per day for females, and the NOAEL was 100 mg/kg bw per day in both sexes.

Developmental toxicity study in rats

A non-GLP compliant *range-finding* study (WIL Research, 2015a) was performed in groups of 8 female rats (Crl:CD(SD)), who were administered 125, 250, 500 and 1,000 mg 3-(1-((3,5-dimethylisoxazol-4-yl) methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-imidazolidine-2,4-dione [FL-no: 16.127]/kg bw per day, suspended in 1% methylcellulose. The control group received vehicle only. Administration was once daily by oral gavage at 10 mL/kg bw from gestation day (GD) 6–20. Mortality, signs of external clinical changes, body weight and food consumption were monitored. On GD 21, laparohysterectomy was performed.

Dosages were analytically confirmed. No maternal mortality or abortions were observed. No incidences of external clinical manifestations or changes in body weight, gravid uterine weight or food consumption were observed. There were also no significant differences in sex ratio, numbers of viable fetuses (no dead fetuses observed), early- or post-implantation losses, number of implantation sites, number of corpora lutea, preimplantation embryo loss or mean fetal weights. There was only one single incidence of an abnormal fetus at 250 mg/kg bw per day.

The study conclusion to use doses of 125, 500 and 1,000 mg/kg bw per day for the full developmental toxicity study is justified.

The full developmental toxicity study was conducted according to GLP and followed OECD Test No 414: Prenatal Development Toxicity Study Guidelines (WIL Research, 2015b). Groups of 25 female rats (Crl:CD(SD) ~ 14 weeks old) were administered 125, 500 and 1,000 mg test compound [FL-no: 16.127]/kg bw per day, suspended in 1% methylcellulose. The control group received vehicle only. Administration was once daily by oral gavage at 10 mL/kg bw from GD 6–20. Twice daily mortality and moribundity were checked, and signs of external clinical changes, body weight and food consumption were monitored. On GD 21, laparohysterectomy was performed, followed by extensive examination of the relevant organs in the dams and the fetuses as indicated in OECD test 414, similar but more extensive than in the range-finding study.

No incidences of maternal mortality, abortion or requirement for killing were reported; two controls and three of the 1,000 mg/kg bw per day females were non-gravid. No significant differences in clinical observations of the dams were reported, including hair loss, dosage reaction, body weight changes, gravid uterus weights or food consumption. At GD 21, there were no significant differences reported for: number of viable fetuses, dead fetuses (zero for all groups), early/late resorptions, post-implantation embryo loss, number of implantation sites and corporal lutea, preimplantation embryo loss, twining rate or sex ratio. Only the body weights of female fetuses in the 125 mg/kg bw per day group were significantly (5.7%) heavier than controls. There were no differences in the incidences of fetal malformations between all groups.

Conclusion

In a developmental toxicity study, according to GLP and OECD test guideline 414, rats were administered oral doses of 125–1,000 mg/kg bw per day of the candidate substance. There were no differences between the treated and control groups. Therefore, there is no concern for developmental toxicity of [FL-no: 16.127] in rats at dose levels up to 1,000 mg/kg bw per day.

Table F.1: Genotoxicity data (*in vitro*)

Chemical name [FL-no]	Test system	Test object	Concentration	Reported result	Reference	Comments
(3-(1-((3,5-Dimethylisoxazol-4-yl) methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2,4-dione) [16.127]	Ames test	*Salmonella* Typhimurium TA98 and TA100	Up to 5 mg/plate[(a)]	Negative	Nucro-Technics (2008a)	Initial screening test
	Ames test	*Salmonella* Typhimurium TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 uvrA	Up to 5 mg/plate[(a)]	Negative	Huntingdon Life Sciences (2010a)	Test in according with OECD Guideline 471. GLP
	Chromosome aberration test	Human lymphocytes	Up to 823.8 μg/mL[(b)] and 1,300 μg/mL[(c)]	Negative	Huntingdon Life Sciences (2010b)	Test in accordance with OECD Guideline 473. GLP

Chemical name [FL-no]	Test system	Test object	Concentration	Reported result	Reference	Comments
Hydrolysis product; S6893	Ames test	*Salmonella* Typhimurium TA98, TA100, TA1535 and TA1537 *E. coli* WP2 uvrA	Up to 5,000 μg/plate[(a)]	Negative	Huntingdon Life Sciences (2009a)	

GLP: good laboratory practice; OECD: Organisation for Economic Co-operation and Development.
(a): With and without metabolic activation.
(b): Without metabolic activation.
(c): With metabolic activation.

Table F.2: Genotoxicity data (*in vivo*)

Chemical name [FL-no]	Test system	Test object	Route	Dose	Reported result	Reference	Comments
[16.127]	Micronucleus test	Mice	Oral by gavage	Up to 2,000 mg/kg bw	Negative	Huntingdon Life Sciences (2010c)	Test in accordance with OECD Guideline 474. GLP

GLP: good laboratory practice; OECD: Organisation for Economic Co-operation and Development.

Table F.3: Toxicity data (*in vivo*)

Chemical name [FL-no]	Species; sex no/group	Route	Doses (mg/kg bw per day)	Duration	Result (mg/kg bw per day)	Reference	Comments
(3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2, 4-dione) [16.127]	Sprague–Dawley rats; 5 female/ dose level	Gavage	0, 5, 15 and 50 mg/kg bw	Single dose, 8 days observation	–	Nucro-Technics (2008b)	Pretesting screening test
	Mouse	Gavage	2,000 mg/kg bw	Single dose, 8 days	–	Huntingdon Life Sciences (2010c)	Range-finding study for mouse micronucleus shows that a single dose of 2,000 mg/kg bw is well tolerated
	Crl:CD(SD) rats: 5 male, 5 female per dose level	Dietary	0, 10, 30 or 100 mg/kg bw per day	28-days	–	Huntingdon Life Sciences (2009b)	Range-finding study
	Crl:CD(SD) rats: 10 male, 10 female per dose level	Dietary	0, 10, 30 or 100 mg/kg bw per day	13 weeks (91 days)	NOEL: 30 mg/kg bw per day NOAEL: 100 mg/kg bw per day	Huntingdon Life Sciences (2010e)	Test in accordance with OECD Test Guideline 408. GLP

GLP: good laboratory practice; NOAEL: no-observed-adverse-effect-level; NOEL: no-observed-effect-level; OECD: Organisation for Economic Co-operation and Development.

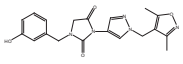
Table F.4: Developmental toxicity data (*in vivo*)

Chemical name [FL-no]	Species; sex no/group	Route	Doses (mg/kg bw per day)	Duration	Result (mg/kg bw per day)	Reference	Comments
[16.127]	Crl:CD(SD) rats: 25 pregnant female/dose level	Oral gavage	0, 125, 500, 1,000 mg/kg bw per day	Gestational; days 6–20	NOAEL: 1,000 mg/kg bw per day	WIL Research (2015b)	Developmental toxicity study. Conducted in accordance to OECD Guideline 414. GLP

GLP: good laboratory practice; NOAEL: no-observed-adverse-effect-level; OECD: Organisation for Economic Co-operation and Development.

Appendix G – Summary of the procedure

Table G.1: Summary of the procedure for the evaluation of individual flavouring substances

FL-no JECFA no CAS no	Union list name	Structural formula	Procedure pathway (A or B)[a]	Class[b] Evaluation procedure path[a]	Toxicological data required	Chronic APET μg/person per day (adult or child)[c]	EFSA conclusion
16.127 2161 1119831-25-2	(3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl) imidazolidine-2, 4-dione)		B	Class III B3: Intake 10 times below TTC	90-day study Developmental toxicity study	850	Studies are available and the margin of safety can be calculated

APET: added portions exposure technique; CAS: Chemical Abstract Service; JECFA: The Joint FAO/WHO Expert Committee on Food Additives.
(a): Are data available to demonstrate that metabolites are to be considered innocuous? Yes: A; No: B.
(b): Thresholds of concern: Class I = 1,800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(c): The highest chronic APET value among adults and children expressed in μg/person per day.

Table G.2: Summary of the evaluation of metabolites

FL-no JECFA no	Union list name/code name		Estimated amount MSDI (EU) μg/capita per day	EFSA status	Cramer class	Comments
Non-Register	S7558[a]	Glucuronide conjugate of [FL-no: 16.127][a]	Not available	Not evaluated as a flavour	III	Conjugate of candidate substance
Non-Register	S5907[a]	Sulfate conjugate of [FL-no: 16.127][a]	Not available	Not evaluated as a flavour	III	Conjugate of candidate substance
Non-Register	S6887[a]	2,3-dihydroxybenzyl derivative of [FL-no: 16.127][a]	Not available	Not evaluated as a flavour	III	Hydroxylated metabolite

JECFA: The Joint FAO/WHO Expert Committee on Food Additives; MSDI: maximised survey-derived daily intake.
(a): See Figure D.1.